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Influence of cosolvents on the hydrophobic surface immobilization topography of *Candida antarctica* lipase B☆

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ABSTRACT

The presence of cosolvents and co-solutes during the immobilization of lipases on hydrophobic supports may influence the extent of lipase immobilization and the long-term catalytic stability of the biocatalyst. *Candida antarctica* B lipase immobilization was examined on a hydrophobic surface, i.e., gold modified with a methyl-terminated, self-assembled alkylthiol layer. Lipase adsorption was monitored gravimetrically using a quartz crystal microbalance (QCM). Lipase activity was determined colorimetrically by following *p*-nitrophenol propionate hydrolysis. Adsorbed lipase topography was examined by atomic force microscopy (AFM). Lipase adsorption from low ionic strength aqueous buffer produced a uniform confluent protein monolayer. Inclusion of 10% (vol) ethanol in the buffer during immobilization resulted in a 33% adsorbed mass increase. Chemically similar cosolvents, all at 10% by volume in buffer, were also individually examined for their influence on CALB adsorption. Glycerol or 1-propanol increased mass adsorption by 10%, while 2-propanol increased mass adsorption by 33%. QCM dissipation values increased threefold with the inclusion of either ethanol or 2-propanol in the medium during lipase adsorption, indicating formation of multilayers of CALB. Partial multilayer formation using 10% ethanol was confirmed by AFM. Inclusion of 10% ethanol in the CALB immobilization buffer decreased the specific activity of the immobilized lipase by 37%. The formation of lipase multilayers in the presence of certain cosolvents thus results in lower specific activity, which might be due to either influences on lipase conformation or substrate active site accessibility.

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1. Introduction

Use of lipases for non-aqueous synthesis is a well-established technique [1–4]. The stereo- and regio-specificity achieved with lipase-catalyzed reactions in many cases is superior to that obtained by conventional chemical approaches. *Candida antarctica* lipase B (CALB) has been particularly useful in this regard [5,6]. Commercial applications, as well as much investigational research, rely on immobilized forms of CALB, such as Novozym 435 [7,8]. Immobilization can impart greater thermal stability and facilitate lipase re-use [9].

It is clear that the nature of the support selected for lipase immobilization influences catalytic performance, as reflected by differences in specific activity, substrate selectivity and thermal stability [10,11]. CALB immobilization on a multitude of support materials has been studied, including polar or hydrophobic polymers [12–17], ion-exchange resins [18], modified controlled-pore glass [19], derivatized silica [20–22], silica gels [23,24], clay [25], and activated carbon [9]. CALB attachment to the support can be through simple adsorption for hydrophobic surface, ion exchange or covalent linkage [26]. Each approach has its own positive and negative attributes that impact enzyme performance. For simplicity and cost-effectiveness, lipase adsorption to a hydrophobic support is preferred in non-aqueous applications [27].

Although much attention has been paid to the influence of the immobilization support on CALB performance, less is known about the optimized conditions of the aqueous phase for CALB adsorption. Protein concentration, solution pH, temperature, and time are known important factors [26]. However, co-solutes may also impact the immobilization process [9,28]. Co-solutes may affect the protein–surface interaction or protein–protein association, which in turn may impact the efficacy of the immobilization process. Several co-solutes have been shown to be effective for increasing covalently immobilized CALB activity [28], but these co-solutes do not have a similar impact on physically adsorbed enzyme (i.e., Novozym 435; unpublished observation, J. Laszlo). CALB has only a

☆ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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small active site lid [29], so interfacial activation by solutes or support would not expected to be a contributing factor in raising its activity level. A greater understanding of how co-solutes influence CALB and its immobilization is therefore needed.

It is common practice to expose a hydrophobic support to a polar organic solvent prior to contacting the support with aqueous lipase [19,22,30–33]. The solvent lowers the surface tension of the support and presumably allows better contact of the lipase solution with the support. In effect, the organic solvent becomes a co-solute of the aqueous immobilization medium. It was shown recently that immobilization of CALB on octyl silica was greatly improved, both in quantity of protein adsorbed and its specific activity, by inclusion of ethanol in the buffer [34]. Ethanol allowed better distribution of CALB throughout the octyl silica pores. Not considered was the influence of the organic solute on the support's hydrophobic surface characteristic. Ethanol would be expected to partition into the octyl layer, essentially making the surface more polar. A comparison of hydrophilic and hydrophobic self-assembled monolayer (SAM) surfaces showed them to equally adsorb CALB, but there was far better activity retention with the hydrophobic surface [35]. This raises the question of whether the presence of co-solutes such as ethanol present during CALB immobilization, on an otherwise hydrophobic surface, impact the amount of lipase adsorbed and its specific activity in the absence of gross architectural restrictions such as support pore size and aqueous media accessibility.

2. Experimental

2.1. Reagents and materials

CALB in solution (commercial name Lipozyme CALB-L) was obtained from Novozymes North America. 1-Undecanethiol (MU) and ethanol were from Sigma–Aldrich. Water was obtained from a Barnstead NANOpure Diamond UV ultrapure water purification system (resistivity 18.2 MΩ cm).

2.2. CALB precipitation and dialysis

CALB from the supplier was separated from its shipping/storage medium by cold acetone precipitation [36]. One part CALB was mixed with four parts ice-cold acetone in a glass tube. Fluid was decanted and the precipitate was rinsed with additional cold acetone. CALB was solubilized at a concentration of 2 mg mL^{-1} in 10 mM potassium phosphate (pH 7.0). The CALB solution was dialyzed (Pierce Slide-A-Lyzer cassettes, 7000 MWCO) 24 h at 4 °C, and then stored on ice until used.

2.3. Self-assembled monolayer (SAM) preparation

The Au surfaces of QCM sensor crystals (Q-Sense) and Au-coated glass slides (Platypus Technologies) were cleaned sequentially by UV/O_3 treatment (15 min), H_2O/NH_4OH/H_2O_2 (5:1:1 by volume) at 70 °C (15 min), another UV/O_3 treatment (15 min), and finished with rinses of water and then ethanol. Au-covered mica (SPI Supplies) was not subjected to the H_2O/NH_4OH/H_2O_2 cleaning step but instead was given water and methanol rinses in between UV/O_3 treatments. Cleaned substrates were immersed for at least 24 h in ethanolic solutions of 10 mM MU to form the hydrophobic, methyl-terminated SAM layer. The SAM surface was rinsed with ethanol and dried under a stream of N_2.

2.4. Protein and hydrolytic activity assays

CALB concentration in solution was determined by the bicinchoninic acid method [37,38], using purified CALB powder (Polium Technologies, Hoffman Estates, IL, USA; stated purity 95%) to prepare calibration standards. Samples and standards were incubated at 37 °C for 30 min, allowed to cool to room temperature, and then their absorbances were measured at 562 nm.

Surface-immobilized CALB specific activity was assessed by following the catalytic generation of the *p*-nitrophenolate anion (15,000 M^{-1} cm^{-1} at 410 nm) from pNPP hydrolysis [39]. One unit (U) of lipase activity produces 1 μmol of product per min. Reactions were conducted in 10 mM potassium phosphate (pH 7.0) containing 1.0 mM pNPP at 27 °C. Color development was linear with time for several minutes. pNPP hydrolysis in the absence of CALB was negligible in this short time period. To immobilize CALB, SAM-modified Au-coated glass slides (25 mm × 75 mm) were wetted with 10% ethanol in buffer and then transferred to a Coplin staining jar containing 20 μg mL^{-1} CALB in buffer (or in 10% ethanol/buffer). The jar was kept in an orbital shaker (90 rpm) for 20 min, and then the slides were transferred to buffer for 5 min to remove loosely adhered protein. Slides were removed from the beaker and residual buffer was wicked from their surface. To create reaction wells on the slides, flat-sided glass O-rings (2.25 cm i.d., 0.5 cm high) were attached to the slides (two O-rings per slide) with vacuum grease. Each O-ring enclosed 4.0 cm^2 of slide surface. The constructed wells were filled with 1.0 mL of 1.0 mM pNPP and the slides were placed in a forced-air orbital shaker operating at 90 rpm and 27 °C. Slides were covered with a Petri dish to minimize fluid evaporation from the wells. At timed intervals, the entire well reaction solution was transferred to cuvettes for photometric analysis of the reaction product concentration.

2.5. Size exclusion chromatographic analysis

Dialyzed CALB (see Section 2.2) was subjected to size exclusion chromatography using a Superose 6 10/300 GL column (Amersham Biosciences) at ambient room temperature. The elution medium (50 mM potassium phosphate, pH 6.5, with or without 10% ethanol cosolvent) flow rate was 0.5 mL min^{-1}. The protein elution profile was monitored by absorbance at 280 nm.

2.6. Quartz crystal microbalance (QCM) measurements

QCM measurements of CALB adsorption to SAM-modified Au surfaces on AT-cut quartz crystals were performed with a Q-Sense D300 system (Q-Sense, Inc., Glen Burnie, MD). The crystal and solution chamber temperature was maintained at 25.0 °C. The QCM with dissipation technique, described in detail elsewhere [40,41], provides information about the amount of adsorbed mass through changes in vibrational frequency (*f*). For rigid films, the Sauerbrey equation [40] can be employed to determine adsorbed mass (Δm):

$$\Delta m = -\left(\frac{C}{N}\right)\Delta f \quad (1)$$

where *C* is the mass sensitivity constant, 17.7 ng cm^{-2} Hz^{-1}, at the primary harmonic (5 MHz) and *N* is the overtone number. The 15 MHz ($N = 3$) overtone was used for quantifying adsorbed mass (ng cm^{-2}). Dissipation values (ΔD), a measure of film rigidity, were also recorded at the 15 MHz overtone.

SAM-modified crystals were assembled into the QCM unit, and then flushed successively with buffer (10 mM potassium phosphate, pH 7.0, 0.2 μm filtered and degassed), 10% (vol) ethanol, and buffer again (5 mL for each step). Buffer was passed into the cell to establish baseline values in liquid. A solution of CALB (20 μg mL^{-1}) in buffer (or 10% organic solvent in buffer) was slowly introduced into the QCM cell, which allowed CALB to adsorb onto the crystal surface under flow conditions for approx. 20 min. The cell was then flushed with buffer. Crystal frequency and dissipation values were continuously monitored in the conventional manner through-

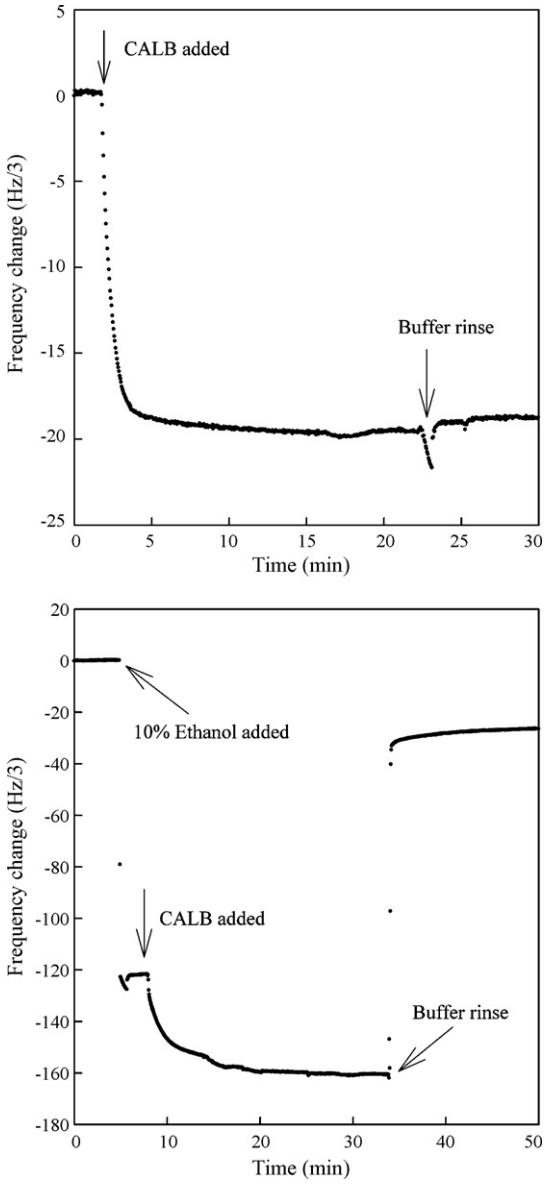

Fig. 1. QCM-D analysis of CALB adsorption to a hydrophobic SAM surface from aqueous buffer (upper graph) or buffer containing 10% by volume ethanol (lower graph).

out the experiment, but Δf and ΔD values were calculated based only on the frequency differences between the initial and final buffer steps in the adsorption regime under non-flowing conditions (Fig. 1).

2.7. Atomic force microscopy (AFM)

CALB adsorbed to SAM-modified Au on mica was imaged in fluid using tapping mode AFM with a multimode Nanoscope IV (Veeco Metrology) instrument equipped with an E scanner and a silicon nitride probe (Veeco NP-S10). The mica was affixed to a steel puck using an epoxy resin, then mounted onto the scanner. An imaging fluid cell was placed over the surface, providing a closed flow system equivalent to the QCM apparatus. Buffer, 10% ethanol in buffer, and CALB solutions were sequentially drawn through the flow cell as described for the QCM (Section 2.5). Multiple 500 nm × 500 nm images (512 samples per scan line) were collected from non-overlapping locations.

Table 1
QCM-D determination of CALB mass adsorption and film rigidity on a hydrophobic surface.

Cosolvent	Frequency change (Hz)[a]	Coupled mass (ng cm^{-2})[b]	Dissipation change ($\Delta D \times 10^{-6}$)
None	56.8 ± 1.0 (6)	335 ± 6	0.4 ± 0.2
Ethanol	75.8 ± 3.2 (5)	447 ± 19[c]	1.2 ± 0.2
1-Propanol	62.9 ± 2.5 (6)	371 ± 15[c]	0.6 ± 0.1
2-Propanol	75.3 ± 5.4 (3)	444 ± 32[c]	1.0 ± 0.2
Glycerol	62.7 ± 2.5 (3)	370 ± 15[c]	0.2 ± 0.2

[a] Mean frequency change and standard deviation at the 15 MHz overtone. Values in parentheses are the number of determinations.
[b] Calculated using Eq. (1).
[c] Coupled mass significantly different ($P < 0.01$) from that obtained in the absence of cosolvent.

3. Results

3.1. QCM-D analysis of CALB adsorption

Modification of Au with long-chain, methyl-terminated alkane thiols produces a hydrophobic surface to which CALB readily adsorbs from low ionic strength media [35]. QCM-D measurements with CALB at a concentration of 20 μg mL^{-1} indicated that maximum adsorption to the surface was achieved within 20 min (Fig. 1). CALB concentrations of 2 and 200 μg mL^{-1} produced the same result, so 20 μg mL^{-1} was considered a saturating concentration for all further experiments. The change in dissipation value (ΔD) with the adsorption of protein layer was at or below 10^{-6}. Therefore, the adsorbed layer was rigidly coupled to the surface and the Sauerbrey equation could be applied for the determination of coupled mass (Table 1). Coupled mass includes water, but for the purposes of this work no distinction between water mass and protein mass was made because the final condition for comparison of coupled mass (i.e., in buffer without cosolvent) was identical and therefore the extent of protein layer hydration likely was as well.

The effect of the presence of cosolvents in the immobilization buffer on the adsorption behavior of CALB was determined by QCM-D. Inclusion of 10% (vol) ethanol increased the amount of CALB adsorbed by 33% compared to that achieved using buffer lacking cosolvent (Table 1). The presence of ethanol during immobilization also increased the dissipation of the film in buffer (no ethanol present) by three-fold after immobilization, indicating that the formed film was less rigid than that of the film produced without cosolvent. The Sauerbrey equation was still employed for calculating adsorbed mass, although it is known to underestimate coupled mass values when the film has some elastic characteristic. Other cosolvents had a variable impact on CALB immobilization, with glycerol and 1-propanol promoting a small increase in adsorption (10%), and 2-propanol providing an increased mass adsorption identical to that generated by ethanol (Table 1). The observed increase in CALB adsorption due to ethanol was also replicated by first forming an adsorbed layer in the absence of ethanol, followed by further treatment with CALB in 10% ethanol (not shown). A similar increase in adsorbed mass was not observed when the adsorbed layer formed from CALB immobilization in buffer was rinsed with 10% ethanol and then with buffer, which indicated that ethanol was not remaining bound to the protein or somehow otherwise influencing the determination of bound CALB.

Washes with 100 mM KCl of the CALB films formed from either buffer or 10% ethanol did not diminish the amount of mass coupled to the SAM surface.

3.2. AFM imaging of adsorbed CALB

CALB films formed on SAM-modified Au were inspected by AFM (Fig. 2). The SAM-modified Au surface consisted of interlocking

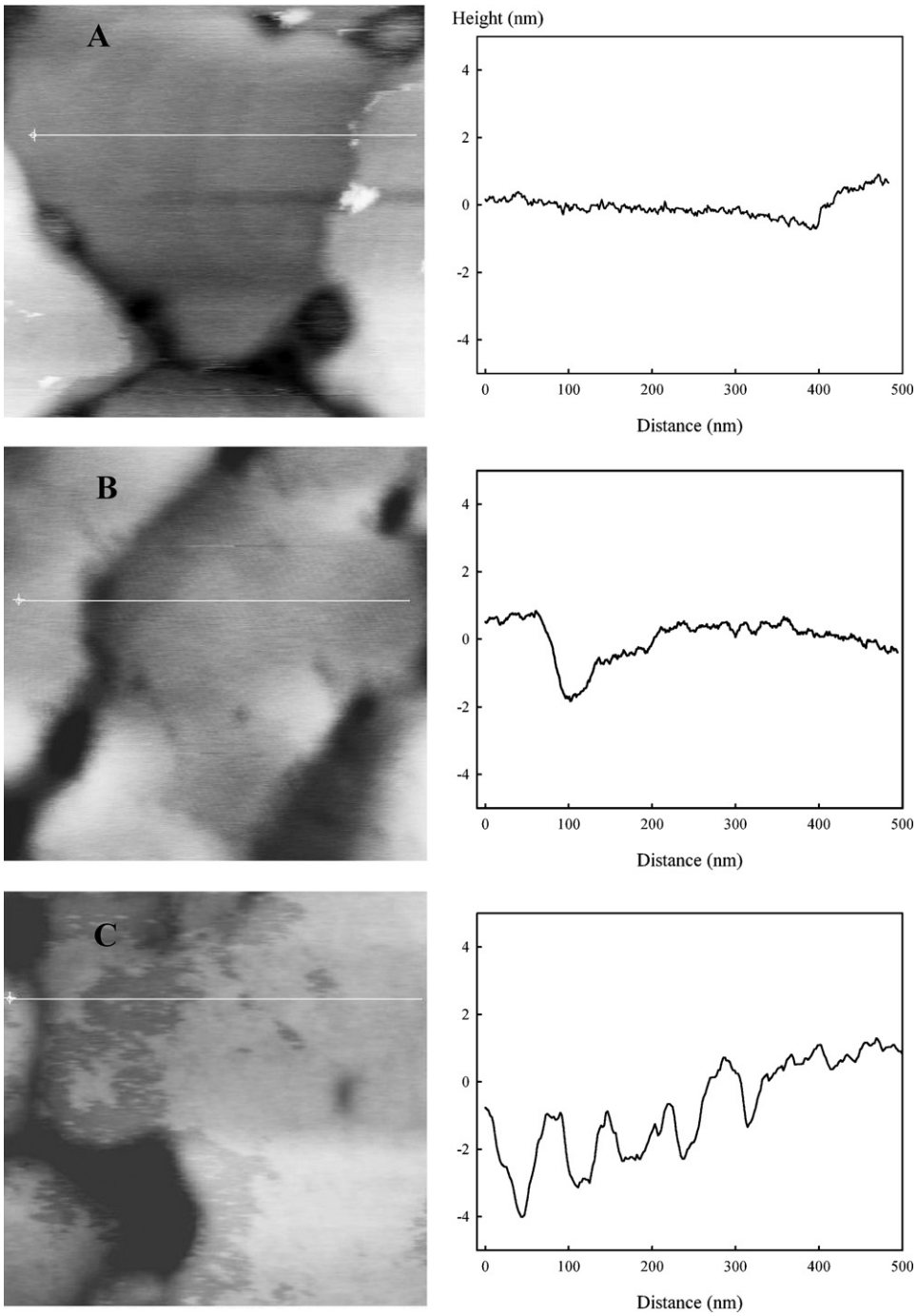

Fig. 2. AFM images (left) and section analysis (right) of (A) the SAM-modified Au/mica surface, (B) CALB adsorbed to a hydrophobic SAM surface from aqueous buffer or (C) from buffer containing 10% by volume ethanol. The dimensions of the images are 500 nm × 500 nm × 10 nm (*X*, *Y*, *Z* dimensions, respectively). The white line superimposed on the images corresponds to the data used for section analysis. The images are not of the same location.

grains (Fig. 2A). Within a grain, the surface was very flat, which provided a large area suitable for imaging adsorbed protein topography. CALB drawn through the cell at a concentration of 20 μg mL^{-1} formed a confluent, uniform-height, protein layer on the SAM surface (Fig. 2B). This film was previously determined to be 5.5 nm high, representing a single monolayer of CALB [35]. The roughness of the CALB-covered surface was not significantly different from that of the SAM surface (Table 2). The inclusion of 10% ethanol in the buffer during CALB immobilization produced a film of more variable height, indicating that a partial formation of multilayered structures had occurred (Fig. 2C). Brighter/whiter areas of the AFM image (Fig. 2C) indicate secondary CALB layer formation atop the

Table 2
AFM surface roughness analysis of CALB on SAM-modified Au/mica.

Surface	Rq (nm)[a]	Ra (nm)[b]
SAM-modified Au/mica	0.157 ± 0.022 (9)	0.124 ± 0.017 (9)
CALB applied from buffer	0.160 ± 0.045 (8)	0.126 ± 0.034 (8)
CALB applied from 10% ethanol	0.304 ± 0.123 (12)	0.236 ± 0.090 (12)

[a] Root mean square (RMS) height deviation from the mean plane for selected 100 nm × 100 nm areas within a grain boundary. Values in parentheses are the number of determinations.

[b] Arithmetic average of the absolute values of surface height deviations measured from the mean plane for selected 100 nm × 100 nm areas within a grain boundary. Values in parentheses are the number of determinations.

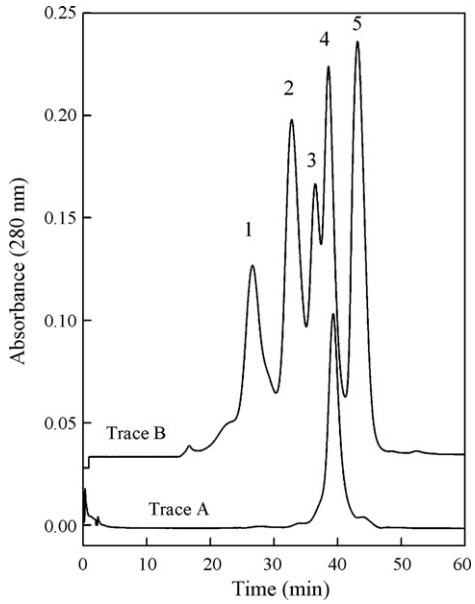

Fig. 3. Size exclusion chromatography of protein in buffer containing 10% ethanol. Trace A is of CALB and Trace B is of molecular weight markers (1) bovine thyroglobulin, 670 kDa, (2) bovine γ-globulin, 158 kDa, (3) chicken ovalbumin, 44 kDa, (4) horse myoglobin, 17 kDa, and (5) vitamin B_{12}, 1.35 kDa.

CALB directly adsorbed to the SAM surface. Its surface roughness was significantly higher ($P < 0.01$) than that of the CALB layer formed in the absence of 10% ethanol (Table 2). Observation of multilayer formation was consistent with the findings from the QCM-D measurements.

3.3. Size exclusion chromatographic analysis

Although CALB is known to occur as a monomer in solution [42], it was deemed prudent to consider whether inclusion of cosolvent in the immobilization medium induced some aggregation that may have contributed to the multilayer formation observed by QCM-D and AFM. Size exclusion chromatography of CALB in the presence of 10% ethanol resulted in a single peak (Fig. 3, Trace A). The absence of higher molecular weight species in the chromatogram indicated that there was no protein aggregation in solution induced by 10% ethanol.

The elution behavior of CALB in buffer, with or without cosolvent, was somewhat anomalous with regard to its apparent size. Based on the size exclusion standards (Fig. 3, Trace B), CALB had an elution time equivalent to an approximately 17 kDa molecule, although it is well known to be 33 kDa in size. That this peak was indeed CALB was confirmed by hydrolytic activity assays and SDS-PAGE. This curious elution behavior does not contradict the conclusion that 10% ethanol had no aggregating effect on the protein in solution. However, the anomalous elution behavior could point to interactions of the protein with the column matrix (a polysaccharide), which might obscure or disrupt weak protein–protein interactions.

Table 3
Immobilized CALB hydrolytic activity.

Immobilization cosolvent	Activity ($U\,cm^{-2}$)[a]	Specific activity ($U\,mg^{-2}$)[b]
None	2.6 ± 0.5 (12)	8.0
Ethanol	2.2 ± 0.3 (8)	4.8

[a] Mean activity (pNPP hydrolysis rate in buffer without cosolvent) and standard deviation from the mean. Values in parentheses are the number of determinations.
[b] Based on coupled mass per cm^2 (Table 1).

3.4. Immobilized CALB hydrolytic activity

CALB did not display significantly different hydrolytic activities when immobilized on a hydrophobic SAM surface in the presence or absence of 10% ethanol (Table 3). However, when compared on a specific activity basis (hydrolytic rate per unit mass of adsorbed CALB), the CALB film formed in the presence of ethanol had a 37% lower value. This indicates that the additional CALB adsorbed by the surface in the presence of ethanol did not proportionally increase the activity of the film.

4. Discussion

The common practice of pre-wetting a hydrophobic support material with an organic solvent prior to or during lipase immobilization permits greater contact of lipase with the support surface and therefore higher amounts of protein adsorption. This effect has been recently highlighted by Blanco et al. [34] with CALB immobilization on octyl silica, a hydrophobic mesoporous support. Inclusion of 10% ethanol produced a twofold increase in the amount of CALB immobilized. The hypothesis offered was that cosolvent enables greater internal pore surface access. The wetting process with ethanol or other cosolvent involves partitioning of the organic molecule into the surface layer of the hydrophobic support, thus making it more hydrophilic. If the cosolvent remains after the wetting step (i.e., is not removed by a subsequent aqueous rinse before the lipase immobilization step), then there may be an impact on the interaction of the lipase with the support surface, which is no longer as hydrophobic.

Prior work demonstrated that CALB binds to hydrophilic and hydrophobic SAM surfaces to a similar extent, but the hydrophobic surface retains much greater activity [35]. The former and present work were performed using non-porous surfaces so that pore access by the lipase was precluded as a concern. Therefore there was an expectation that a cosolvent such as ethanol would not increase the amount of CALB bound. Nonetheless, cosolvents did increase CALB binding, although not to the twofold extent observed by Blanco et al. with octyl silica. Blanco et al. surmised from adsorption isotherms that CALB forms a monolayer on octyl silica regardless of the presence of cosolvent. The present study demonstrates that cosolvent ethanol inclusion does lead to multilayer CALB formation. Thus the positive influence of ethanol cosolvent for immobilizing the lipase on octyl silica may be attributed to two different but complementary effects: greater pore access and lipase multilayer formation.

The observed decreased specific activity of CALB immobilized in the presence of 10% ethanol indicates that multilayer formation may not be conducive to achieving maximum performance from the catalyst. This finding is at odds with those of Blanco et al. who established that ethanol inclusion raises sixfold the specific activity of CALB on octyl silica. Reconciling these divergent findings will require further work. While it may be tempting to speculate that activity loss in multilayered CALB films is due to decreased substrate access, it should be noted that cross-linked enzyme aggregates of CALB retain high pNPP hydrolysis rates; some even show enhanced activity compared to the native monomeric enzyme [36]. CALB aggregation alone does not result in hydrolytic activity loss. The aggregation state of CALB on methyl methacrylate resin also has been suggested to play a role in the activity of CALB [16]. Well ordered CALB multilayers may thus have a specific activity different from that of unorganized aggregrates. It is unclear at this time whether this effect is due to mass transfer influences or conformational changes in CALB within multilayers.

The now-standard approach of lipase immobilization on hydrophobic supports provides stabilization and, in many cases, hyperactivation [43]. Hydrophobic residues around the active site

opening or "lid" are believed to be where the lipase interacts with the hydrophobic surface of the support [44]. Simple physical adsorption of CALB to a hydrophobic surface provides better activity than various multipoint covalent attachment approaches for most non-aqueous applications [22,45,46]. The inclusion of certain cosolvents during CALB immobilization appears to result in subtle changes in the adsorption process by altering protein–support and protein–protein interactions.

5. Conclusions

CALB forms a closely packed monolayer on a hydrophobic surface when the surface is pre-wetted with ethanol as a cosolvent in an aqueous buffer, but with the immobilization conducted without ethanol present. Ethanol and 2-propanol induce significant multilayer formation that adversely impacts the specific activity of the resultant catalytic film. These findings suggest a preferred protocol for CALB immobilization that should include the sequential steps of wetting the support first in cosolvent-containing buffer, followed by a rinse to remove cosolvent (without drying of the support surface), and then application of lipase from cosolvent-free buffer to provide full monolayer coverage.

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